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Chloroplast 16S rDNA Sequences and Phylogenetic Relationships of Fern Allies and Ferns

JAMES R. MANHART
Department of Biology, Texas A&M University, College Station, TX 77843-3258

ABSTRACT.—Chloroplast 16S rDNA sequences from the charophyte algae *Spirogyra* and *Coleochaete*; the bryophytes *Marchantia* and *Sphagnum*; the fern allies *Equisetum, Isoetes, Selaginella, Lycopodium, Tmesipteris* and *Psilotum*; the eusporangiate ferns *Angiopteris, Botrychium,* and *Ophioglossum*; the leptosporangiate ferns *Lydogium* and *Doodia*; the gymnosperms *Ephedra* and *Juniperus*; and six angiosperms (two monocots and four dicots) were analyzed with the parsimony algorithm PAUP to infer phylogenetic relationships. The charophytes were the designated outgroup and the bryophytes were at the base of the land plant clade. Relationships of most of the fern allies and *Angiopteris* were unresolved but a clade containing *Psilotum* + *Tmesipteris* (Psilotales) was sister to a clade composed of *Botrychium* and *Ophioglossum* (Ophioglossales). A leptosporangiate fern clade was strongly supported, and this clade grouped weakly with *Angiopteris*. The *Selaginella* 16S rDNA sequence is apparently changing at a faster rate than the other sequences as evidenced by the extremely long branch associated with *Selaginella*. The effect of this long branch on the overall tree topology was tested by removing *Selaginella* from a subsequent analysis. Trees produced without *Selaginella* weakly supported the placement of *Lycopodium* at the base of the vascular plants clade but *Isoetes* grouped weakly with the seed plants. Removal of *Selaginella* also resulted in higher bootstrap values and decay indices for the majority of the clades.

The determination of phylogenetic relationships of the "fern allies," the Psilophyta, Lycophyta, and Sphenophyta, has proven to be elusive. Taxonomically, the fern allies are generally considered to be positioned between the bryophytes and ferns but formal analyses of morphological and molecular data sets only partially resolve their relationships. In a cladistic analysis of morphological characters, Bremer et al. (1987) placed *Psilotum* at the base of vascular plants, the lycopsids branched off next, then *Equisetum,* and then the ferns. However, the portion of the clade containing the fern allies was constructed with only four morphological characters and it is not reasonable to expect that a robust phylogeny containing highly divergent taxa can be produced from such a small data set. Conversely, fossil evidence (Knoll and Rothwell, 1981; Stewart, 1983; Gensel and Andrews, 1984), characters relating to male gametogenesis (Garbary et. al., 1993), and chloroplast genome structures (Raubeson and Jansen, 1992) support the hypothesis that the lycophytes are at the base of the vascular land plant clade.

Mishler et al. (1994) analyzed green plant molecular and morphological data sets separately and together, using parsimony algorithms. Some vascular land plants were included, with the fern allies represented by *Equisetum, Lycopodium, Lycopodiella,* and *Selaginella.* The analysis of "general" morphological characters placed the lycophytes sister to a clade containing *Equisetum,* ferns,

and gymnosperms (Mishler et al., 1994). The only fern allies in the molecular data sets of nuclear rDNA sequences were *Psilotum* and *Equisetum,* and no fern sequences were included. The analyses of 18S rDNA sequences only, and combined morphological and molecular data sets both placed *Psilotum* and *Equisetum* in a clade with seed plants (Mishler et al., 1994).

DNA and derived amino acid sequences of the *rbcL* gene from selected photosynthetic bacteria, green algae, bryophytes, fern allies, ferns, and seed plants were used to construct phylogenetic trees (Manhart, 1994). Generally, the trees produced by these analyses contained clades that are not supported externally by other forms of data (see above) or internally by bootstrap and decay analyses. For example, although the lycophytes grouped together as expected, they were the sister group to the seed plants. This placement of the lycophytes also occured in an analysis that treated *rbcL* sequences as "string" data (Albert et al., 1994). Some clades that were strongly supported by the *rbcL* sequences (Manhart, 1994) included the eusporangiate ferns (*Ophioglossum* + *Botrychium*) and the leptosporangiate ferns, (*Marsilea* + *Lygodium*). Surprisingly, *Psilotum* was in a clade with the Ophioglossales and the leptosporangiate ferns, and this relationship was supported in decay analyses (Manhart, 1994). Within this clade, the placement of *Psilotum* with the Ophioglossales was weakly supported. *Angiopteris* did not group with either the Ophioglossales or the leptosporangiate ferns. In general, relationships of the bryophytes, fern allies (with the exception of *Psilotum*), and *Angiopteris* were unresolved (Manhart, 1994) which indicates that *rbcL* sequences may be too divergent to test phylogenetic relationships among major groups of green plants.

Penny and O'Kelly (1991) pointed out the importance of analyzing several sequences in the construction of phylogenetic trees and testing these trees for corroboration with data from non-molecular sources. The noncongruence of many of the relationships of fern allies indicated by the analysis of *rbcL* sequences with those derived from other evidence indicates the need for the inclusion of sequences from other genes. A logical course to elucidate deep branches in green plant phylogenies is to use a gene less divergent than *rbcL.* The chloroplast *16S rRNA* gene is less divergent than *rbcL* and has been used to construct phylogenetic trees at very broad levels, to include cyanobacteria and plastid sequences (Giovannoni et al., 1988; Woese, 1987; Urbach et al., 1992). Mishler et al.'s (1992) analysis of 370 bp of the chloroplast *16S* and *23S rRNA* genes from a diverse sampling of 11 bryophytes indicated that these sequences may be able to resolve deep branches of land plant phylogenies.

In this study, chloroplast *16S rRNA* gene sequences were analyzed to test phylogenetic relationships of land plants, particularly the fern allies, indicated by analyses of the *rbcL* sequences (Manhart, 1994) and those derived from other data sets (Knoll and Rothwell, 1981; Raubeson and Jansen, 1992; Mishler et al., 1994).

Materials and Methods

PCR Amplification.—Polymerase Chain Reaction (PCR) amplification of the 16S rDNA gene used the 5′ and 3′ primers (Table 1). The reaction mixture

TABLE 1. Primers used to generate (5′ and 3′) and sequence 16s rDNAs. Numbers indicate position of primer relative to 5′ end of 16S rDNA, 3′ and primers with R are reverse primers.

Primer	Sequence
5′	GATCCTGGCTCAGGATGAACG
20	CTGGCTCAGGATGAAC
33	GGCTCAGGATGAACGCTGG
250	GCGTCTGATTAGCTAGTTGG
250R	CCAACTAGCTAATCAGACGC
500	GTGCCAGCAGCCGCGG
500R	CCGCGGCTGCTGGCAC
650	GCAGAGGGAATTTCCGGT
695R	TTCGCCGTTGGTGTTCTT
750	GACGAAAGCTAGGGGAGCG
750R	CGCTCCCCTAGCTTTCGTC
896R	CCCCCGTCAATTCCTTTG
1050	GTGCATGGCTGTCGTCAGCT
1050R	AGCTGACGACAGCCATGCAC
1230	GAGGAAGGTGAGGATGACGT
1230R	ACGTCATCCTCACCTTCCTC
1400	GGAATCGCTAGTAATCGC
1400R	GCGATTACTAGCGATTCC
1517	AAGGTAGAGCTAGTGAC
3′	AAGGAGGTGATCCAG

consisted of 2.5 units of Taq polymerase from Promega (Madison, WI), buffer supplied by Promega, 0.2 mM dNTP's, 0.1 to 1 μg total DNA, and 50 picomoles of each primer. Double-stranded DNAs were produced by running reaction mixtures in a Perkin Elmer Cetus DNA Thermal Cycler as follows: 1 cycle of 4 min at 94°C, 2 min at 45°C, 3 min at 72°C and 30 cycles of 1 min at 94°C, 2 min at 45°C, 3 min at 72°C. Single-stranded DNAs were produced by adding 10 μl of the double-stranded DNAs to fresh reaction mixtures with each of the primers individually (Kaltenboeck et al., 1992). These reactions were run as for double-stranded DNA except with 20 cycles rather than 30 cycles of amplification in the last step. PCR products were verified by running through 1% agarose gels. DNAs for sequencing were purified using a QIAquick PCR purification kit (Qiagen) as described in instructions.

SEQUENCING.—Sequences were determined from double-stranded cloned DNA and single-stranded amplified DNA by dideoxy-sequencing as described in the Sequenase manual (Version 2.0) using the primers in Table 1. It was determined that better quality sequence was consistently obtained by using dITP instead of dGTP in the reaction mixtures. The sequences were assembled using the GelAssemble program of the GCG DNA analysis package (Devereux et al., 1984). A typical sequencing strategy is shown in Fig. 1 where most of the gene was sequenced on both strands. Problem areas were always sequenced either on both strands or two or more times on the same strand. Sequences were submitted to GenBank (Table 2). Sequences were aligned using the Pileup program of the GCG DNA analysis package (Devereux et al., 1984) and manually.

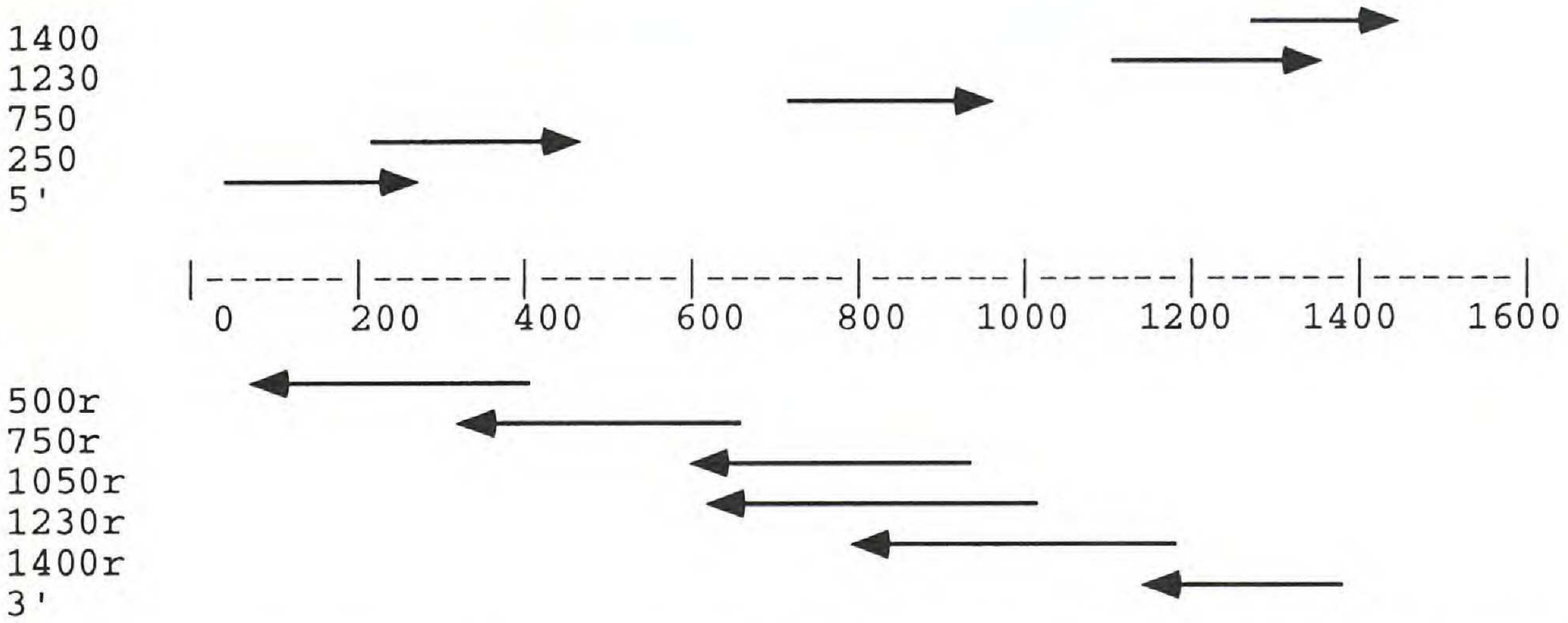

FIG. 1. Sequencing strategy for sequencing *16S rRNA* gene in *Angiopteris*. Arrows indicate beginning and ending points of sequence obtained with primers listed on left.

The complete aligned data set in NEXUS format is available via email request to J-MANHART@TAMU.EDU or by sending a formatted Macintosh floppy disk to the author.

ANALYSIS.—The taxa in Table 2 represent a subset of the taxa included in the *rbcL* analysis (Manhart, 1994). Unlike the *rbcL* gene (Manhart, 1994), alignment of the 16S rDNA sequences required the postulation of insertions and deletions (indels). It is possible to remove these regions from the analysis but this was not done because the *16S rRNA* gene changes very slowly or not at all over most of its length and the removal of all these regions would remove most of the phylogenetic signal. Insertions unique to one taxon are not informative and were not included in the analyses. Regions of sequence that were present in two or more taxa and absent in others were treated as "missing." DNA sequences were analyzed with the computer program PAUP version 3.1.1 (Swofford, 1993) using the "heuristics" option, with collapse of zero-length branches, MULPARS option in effect, random sequence addition with 100 repetitions, and "TBR" branch swapping. The charophytes *Coleochaete* and *Spirogyra* were the designated outgroup taxa. Internal support of the trees (Figs. 2 and 3) was determined by decay analysis (Bremer, 1988; Mishler et al., 1991; Mishler et al., 1994) and bootstrap analysis with 100 replicates (Felsenstein, 1985). Decay analyses to +5 steps in strict consensus trees were run using the settings above but with 10 repetitions of random sequence addition. In addition, 100 Bootstrap repetitions (Felsenstein, 1985) were run using the same settings as above but with simple sequence addition. Alternative topologies for Fig. 2 were investigated using MacClade version 3.01 (Maddison and Maddison, 1992). An identical set of analyses was run with *Selaginella* removed from the data set (Fig. 3). Trees generated in PAUP were saved as PICT files and modified in Canvas 3.0 to produce Figs. 2 and 3.

RESULTS AND DISCUSSION

The region analyzed was 1474 bp in length and includes most of the 16s sequence; 5′ and 3′ primer sequences were not included in the data set. One

TABLE 2. Taxa included in the analyses. References are given for sequences taken from the literature. Sources of material, voucher information, and method of generating DNA for sequencing (cloned fragments, PCR with indicated primers, or a combination of both) are given for new sequences. GenBank/EMBL accession numbers are given for new and unpublished sequences. The acronyms TAMU and ETSU refer to the herbaria of Texas A&M University and Eastern Tennessee State University, respectively, whereas UTEX refers to the culture collection of the University of Texas at Austin. For species indicated with an asterisk (*), more information is available in the appendix to Hasebe et al. (1995), elsewhere in this volume.

Angiopteris evecta (G. Forst.) Hoffm.*, *Nagata* on *20 Dec 1988* (TAMU), 5′ and 3′ primers, GenBank #U24580
Botrychium biternatum (Sav.) Underwood*, *Wilson 5979* (TAMU), 5′ and 3′ primers, GenBank #U24581
Cibotium glaucum (J. E. Smith) Hook. & Arn.* 5′ and 3′ primers, GenBank #U24582
Coleochaete orbicularis Pringsheim, Obtained from L. Graham, 3.2 kb *Hind*III fragment, 5′ and 3′ primers, GenBank #U24579
Daucus carota L., *X73670* (F. Manna, unpublished)
Doodia maxima J. Smith*, 5′ and 3′ primers, GenBank #U24583
Ephedra trifurca Torr., *Hoshaw* on *28 Mar 1992* (TAMU), 5′ and 3′ primers, GenBank #U24584
Equisteum arvense L., *Manhart 05/29/87-1* (TAMU), 5′ and 3′ primers, GenBank #U24593
Glycine max (L.) Merr. (von Allmen and Stutz, 1988)
Isoetes melanopoda Gay & Durieu, *Manhart 03/10/88-1* (TAMU), 5′ and 3′ primers, GenBank #U24585
Juniperus virginiana L., *Northcliffe* on *10 Mar 1991* (TAMU), 5′ and 3′ primers, GenBank #U24586
Lycopodium digitatum Dillenius ex A. Braun, *Manhart 06/12/88-1* (TAMU), 5′ and 3′ primers, GenBank #U24587
Lygodium japonicum (Thunb.) Sw., *Manhart 07/31/91-1* (TAMU), 5′ and 3′ primers, GenBank #U24588
Marchantia polymorpha L. (Ohyama *et al.*, 1986)
Nicotiana tabacum L. (Shinozaki *et al.*, 1986)
Ophioglossum engelmannii Prantl.*, 5′ and 3′ primers, GenBank #U24589
Oryza sativa L. (Hiratsuka *et al.*, 1989)
Osmunda cinnamomea L. var. *fokiensis* Copel.*, 5′ and 3′ primers, GenBank #U24594
Pisum sativum L. (Cerutti and Jagendorf, 1991)
Psilotum nudum (L.) P. Beauv., *Manhart 04/06/88-1* (TAMU), 5′ and 3′ primers, GenBank #U24590
Selaginella apoda (L.) Spring, *Manhart 04/06/88-2* (TAMU), 5′ and 3′ primers, GenBank #U24591
Sphagnum palustre L., *Renzaglia 752* (ETSU), 5′ and 3′ primers, GenBank #U24592
Spirogyra maxima (Hassall) Kützing, *UTEX 2495*, 15.0 kg *Cla*I fragment (Manhart *et al.*, 1990), GenBank #U24596
Tmesipteris oblanceolata Copel.*, 5′ and 3′ primers, GenBank #U24595
Zea mays L. (Schwarz and Koessel, 1980)

region of ambiguous alignment was encountered. This was a four bp region where there were possibly separate insertions in *Tmesipteris* and *Selaginella* sequences relative to the other sequences. Removal of this region had no effect on tree topologies and the results reported here included this region. There were two indels that were phylogenetically informative. One was a deletion found in gymnosperms and angiosperms, the other was an insertion unique to *Psilotum* and *Tmesipteris*. These indels were not included in the analyses because they would have had no effect on tree topology (Figs. 2 and 3). If they had been included, the only result would have been a slightly higher consistency index, because there is no homoplasy in these two characters. Future

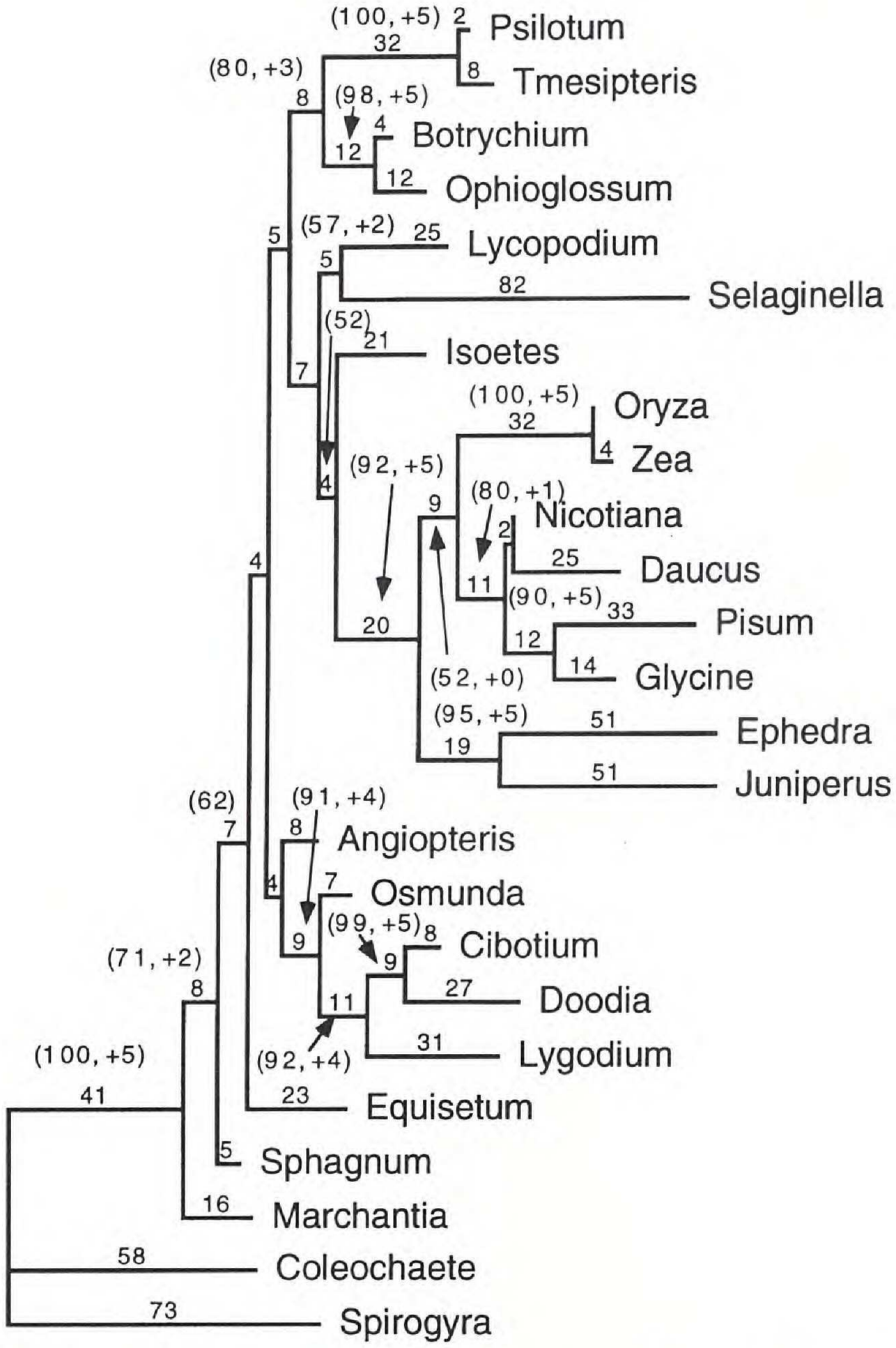

FIG. 2. One of 18 shortest trees. Branch lengths, bootstrap values and decay indices are above each branch with the last two in parentheses. Bootstrap values less than 50 are not given, +0 indicates groups found in all shortest trees. Consistency index excluding uninformative characters=0.48, retention index=0.56.

analyses that combine molecular and phenotypic data should probably include these indels as separate characters. The heuristic search found 18 shortest trees 859 steps in length when *Selaginella* was included in the data set. Fig. 2 is one of the trees, which varied in their placement of *Isoetes, Angiopteris, Equisetum, Nicotiana,* and *Daucus.*

The placements of *Marchantia* at the base of the tree and *Sphagnum* sister to vascular plants were moderately supported by both bootstrapping and decay indices (Fig. 2), and correspond to phylogenies based on morphological characters (Mishler and Churchill, 1985; Bremer et al., 1987), partial 16S and 23S rDNA sequences (Mishler et al., 1992), and combined morphological and molecular characters (Mishler et. al., 1994). In an analysis of *rbcL* sequences (Man-

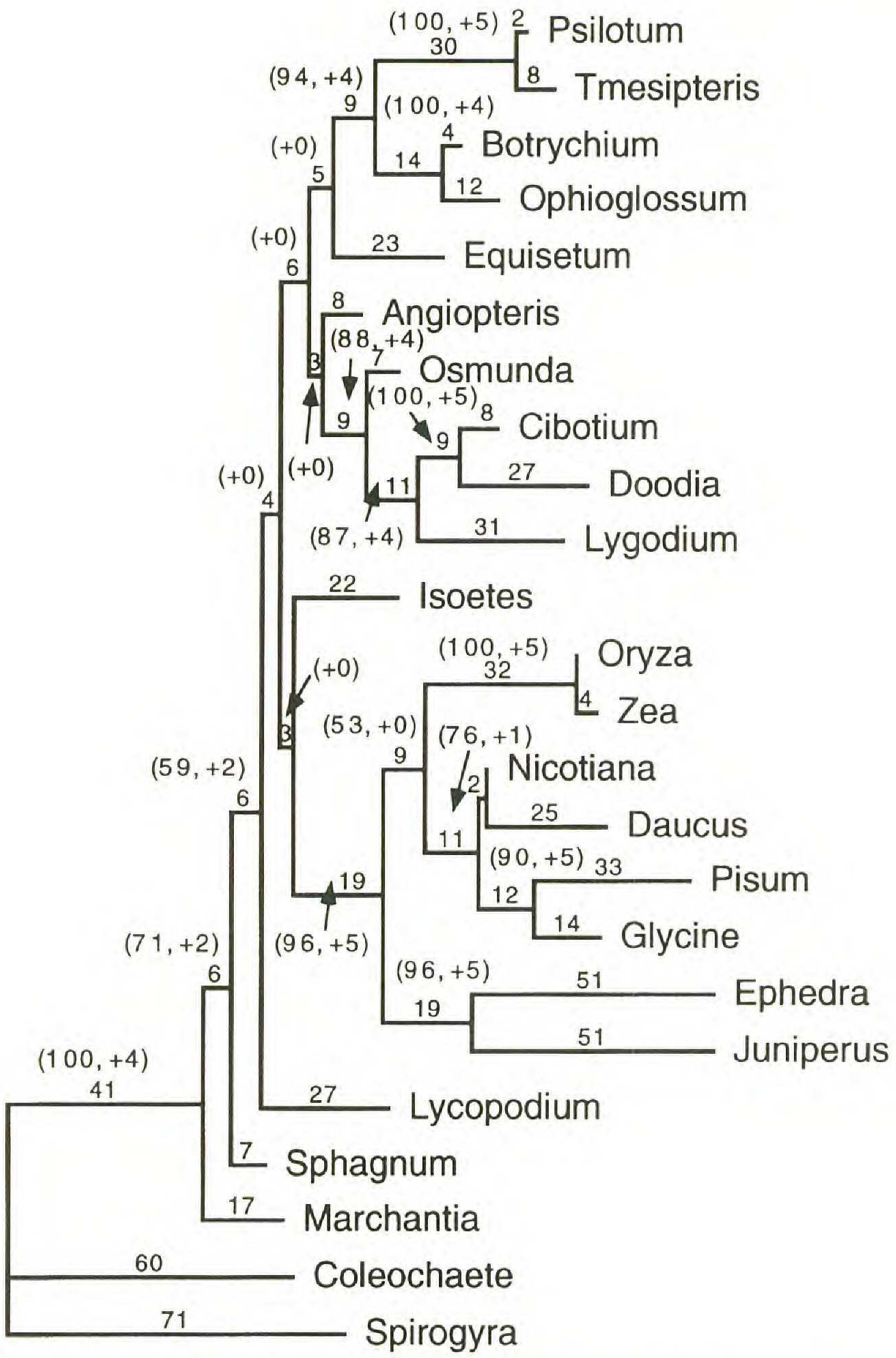

FIG. 3. One of 3 shortest trees with *Selaginella* removed from the data set. Branch lengths, bootstrap values, and decay indices are indicated as in Fig. 2. Consistency index=0.50, retention index=0.58.

hart, 1994), the bryophytes were scattered more or less randomly among the fern allies and *Angiopteris*. Whereas a broader sampling of bryophyte sequences will be required to adequately evaluate the usefulness of the *16S rRNA* gene, this investigation gives a preliminary indication that these sequences may be useful in testing hypotheses of bryophyte relationships.

Equisetum is at the base of the vascular plant clade (Fig. 2) but this placement is not supported by either bootstrapping or decay indices. The placement of *Angiopteris* sister to the leptosporangiate ferns (Fig. 2) is also not supported. Moving *Angiopteris* sister to the base of the clade containing the Ophioglossales + Psilotales added only two steps to the tree in Fig. 2. However, placing *Angiopteris* sister to the Ophioglossales added nine steps. There are apparently no taxa in the 16S analyses to which *Angiopteris* is closely related, which

indicates its origin was separate from the other ferns or that *Angiopteris* diverged from the other ferns very early in their evolution. The relationships of *Equisetum* and *Angiopteris* were also unresolved by analysis of *rbcL* sequences (Manhart, 1994).

The 16S tree (Fig. 2) placed *Isoetes* sister to seed plants and *Lycopodium* and *Selaginella* sister to *Isoetes* and seed plants. The former relationship was weakly supported and the latter unsupported by bootstrapping and decay indices. The placement of *Lycopodium* and *Selaginella* in the same clade is also weakly supported (Fig. 2). Placing *Isoetes* at the base of the clade containing *Lycopodium* and *Selaginella* did not add any steps but placing all the lycophytes at the base of the vascular plants, their expected position based on fossil data and chloroplast DNA structures, added nine steps to the tree in Fig. 2. Trees constructed from *rbcL* sequences (Manhart, 1994) also weakly supported the placement of *Lycopodium* sister to *Selaginella* and in the same clade as *Isoetes,* and this clade sister to seed plants. The placement of the lycophytes together is supported by morphological characters (Mishler and Churchill, 1985) and chloroplast genome structure (Raubeson and Jansen, 1992). The placement of the lycophytes at the base of the vascular plant clade results in additional steps in both 16S (Fig. 2) and *rbcL* (Manhart, 1994) trees and is hence not supported by either gene sequence data set.

The clades containing [*Botrychium* + *Ophioglossum*] and [*Psilotum* + *Tmesipteris]* were strongly supported in the 16S analysis, and the placement of these clades as sister groups was moderately supported (Fig. 2). The clade containing the leptosporangiate ferns was also strongly supported but their relationship to the other land plant clades was unclear (Fig. 2). The placement of *Osmunda* at the base of the leptosporangiate fern clade corresponds to its placement in analyses of *rbcL* sequences (Hasebe et al., 1995). Placement of [leptosporangiate ferns + *Angiopteris*] sister to [Ophioglossales + Psilotales] added three steps to the tree in Fig. 2. The inclusion of more Ophioglossales and leptosporangiate fern 16S sequences may help to determine if they are sister groups. These results are similar to those obtained from *rbcL* sequences (Manhart, 1994), with some minor differences. In the *rbcL* trees, the placement of the Ophioglossales with *Psilotum* was weakly supported and the grouping of these taxa with the leptosporangiate ferns was moderately supported (Manhart, 1994). However, *Tmesipteris* was not included in the *rbcL* analyses. The placement of Psilotales with the Ophioglossales is also supported by nuclear rDNA sequences and subsequent analyses of *rbcL* sequences that included *Tmesipteris* (P. Wolf, pers. comm.).

There is some indication that *Selaginella* chloroplast gene sequences are evolving at a faster rate than those in other land plants because the longest branch in both the 16S (Fig. 2) and *rbcL* (Manhart, 1994) trees was that leading to *Selaginella.* This relatively rapid rate of sequence evolution in *Selaginella* may be responsible for some of the problems encountered in determining lycophyte phylogenetic relationships from chloroplast gene sequences. Bousquet et al. (1992) pointed out that unweighted parsimony methods should not be used in constructing phylogenies from sequences with extensive rate hetero-

geneities. A set of analyses was done with *Selaginella* removed from the data set to determine if the apparently relatively high rate of sequence evolution in *Selaginella* had any affect on tree topology.

Three shortest trees that differed only in dicotyledon relationships were found when *Selaginella* was removed from the data set (Fig. 3). This had a marked effect on the position of *Lycopodium,* which was placed at the base of the vascular plants (Fig. 3). However, this placement was also not supported by bootstrapping or the decay index. *Isoetes* remained sister to the seed plants but this grouping was not supported by bootstrapping and the decay index (Fig. 3). *Equisetum* was sister to [Ophioglossales + Psilotales] rather than at the base of the vascular plant clade (Fig. 2). This relationship was not supported by bootstrapping or decay indices (Fig. 3). The bootstrap values and decay indices for the clades containing [Ophiglossales + Psilotales] and the leptosporangiate ferns (Fig. 3) were increased by removing *Selaginella,* but there was no support for the placement of [Ophioglossales + Psilotales] and the leptosporangiate ferns as sister groups (Fig. 3). In summary, removing *Selaginella* from the data set produced trees with a slightly higher consistency index (0.50 vs. 0.48), and there was stronger support for some clades relative to the trees produced with *Selaginella* (Fig. 2), but there were no significant improvements in resolution of relationships of the lycophytes, *Equisetum,* and *Angiopteris.*

The use of 16S sequences to construct trees containing major groups of land plants (Figs. 2 and 3) resulted in some apparent improvements over trees produced from *rbcL* sequences (Manhart, 1994). The bryophytes are at the base of the 16S trees rather than scattered as in the *rbcL* trees. However, the placements of the lycophytes, *Equisetum,* and *Angiopteris* are uncertain in trees constructed from either gene and both these molecular data sets place the lycophytes sister to the seed plants, a grouping unsupported by morphological, fossil, and chloroplast genome comparisons. It is possible that the inclusion of more bryophyte and lycophyte sequences in the 16S data set may help resolve lycophyte relationships. However, it does not appear likely that analyses of 16S sequences alone, even with expanded sampling and other methods of analysis, will result in a completely resolved phylogeny of land plants. This much desired result will probably require the gathering of additional morphological and molecular data sets and an expanded, combined analysis as in Mishler et al. (1994).

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